Chemiluminescent enzyme immunoassay for detection of PCR-amplified enterotoxin A from *Clostridium perfringens*[1]

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Abstract

A PCR protocol was developed for the rapid and specific detection of *Clostridium perfringens* strains harboring the enterotoxin A gene in artificially contaminated ground beef. A biotinylated primer pair was designed for amplification of a 750 bp fragment of the *C. perfringens* enterotoxin A gene. A combination of 4 h enrichment incubation and nucleic acid extraction, followed by 2 h of PCR amplification allowed detection at levels below 10 CFU of freshly grown cells in raw and cooked beef samples. PCR amplified products were confirmed by a Southern hybridization assay using a digoxigenin-labeled internal probe, and two hybridization ELISA protocols (PCR-ELISA) applying a streptavidin capture step for the hybridized PCR products. Both enzyme immunoassays utilized chemiluminescent detection with Lumiphos 530™ as substrate, after hybridization to an internal digoxigenin-labeled probe or a 5′ conjugated alkaline phosphatase-labeled probe. The PCR-ELISA resulted in faster confirmation of the PCR products while providing a level of sensitivity comparable to Southern hybridization, and has potential for development into an automated method.

Keywords: Chemiluminescence; Enterotoxin A; *Clostridium perfringens*; PCR amplification

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[1] Mention of brand or firm names does not constitute an endorsement by the US Department of Agriculture over others of a similar nature not mentioned.

1. Introduction

The polymerase chain reaction (PCR) is finding increasing applications in diagnostic food microbiology by providing specific and sensitive identification of a number of foodborne pathogens. The technique has been adapted to diagnostic food microbiology using combinations of centrifugation and filtration of food homogenates, DNA extraction and purification (Baez and Juneja, 1995a; Bej et al., 1994; Gannon et al., 1992; Giesendorf et al., 1992; Gustafson et al., 1992; Hill et al., 1991; Lampel et al., 1990), immunomagnetic separation (Fluit et al., 1993; Skjerve et al., 1990), and the use of pre-enrichments for amplification of the number of cells and therefore target sequence(s) (Baez and Juneja, 1995a; Bej et al., 1994; Gannon et al., 1992; Gustafson et al., 1992; Koch et al., 1993; Niederhauser et al., 1992).

The increased adaptability of PCR as a diagnostic detection tool has brought the demand for equally rapid methods for detection of the amplified PCR products. Conventional methods such as ethidium bromide stained-agarose gel electrophoresis and Southern hybridization have limitations in the low sensitivity and lack of confirmation of the PCR amplified products as in the case of ethidium bromide staining, or require time consuming procedures as in the case of Southern hybridization.

The introduction of solution hybridization ELISA for the analysis of PCR amplified products has provided a semi-quantitative tool of superior sensitivity when compared to ethidium bromide staining, and less cumbersome and faster than Southern hybridization (Alard et al., 1993; Chinsangaram et al., 1993; Sabbatini et al., 1993; Soumet et al., 1995; Yang et al., 1993). The solution hybridization ELISA or PCR-ELISA also provides the capability for confirmation of the amplified DNA by incorporation of capture probes or internal probes that will hybridize to the specific amplified sequence. Solution hybridization reactions can also be adapted to a 96-well format allowing for the processing of a large number of samples.

In this study, we report a PCR-based protocol specific for enterotoxigenic *Clostridium perfringens* in ground beef combined with a chemiluminescent enzyme immunoassay for detection and confirmation of the amplified PCR products.

2. Materials and methods

2.1. Bacterial strains

Clostridium perfringens strains NCTC 8238, NCTC 8239, and ATCC 10288, obtained from our culture collection, were used for enterotoxin detection. Non-enterotoxigenic *C. perfringens* strains ATCC 3624 and FD-1 were used as negative

controls. The potential for enterotoxin production and the presence of the enterotoxin gene were confirmed by a reversed passive latex agglutination assay (SPET-RPLA) (Oxoid Inc., Columbia, MD) and by PCR amplification, respectively. In the latter, the oligonucleotide primer pair ENTPS/ENTNS, shown in Table 1, was used for amplification of the 363 bp enterotoxin fragment (data not shown). Stock cultures were maintained in cooked meat medium (CMM; Difco Laboratories, Detroit, MI), and stored at 4°C throughout the course of the study. Vegetative cell cultures were grown by inoculating 0.1 ml of the stock culture into 10 ml of freshly prepared fluid thioglycollate (FTG) medium (Difco Laboratories, Detroit, MI). The inoculated medium was heat-shocked at 75°C for 20 min, followed by aerobic overnight incubation at 37°C. For determination of the number of vegetative cells, the cultures were serially diluted in 0.1% (w/v) peptone-water and plated onto Tryptose-Sulfite-Cycloserine (TSC) agar overlaid with an additional 10 ml of TSC agar (Hauschild and Hilsheimer, 1974). The plates were incubated overnight at 37°C in anaerobic jars (BBL GasPack Anaerobic Systems, Beckton Dickinson, Cockeysville, MD).

2.2. Meat preparation and enrichment procedure

Ground beef was obtained from local retail markets. Individual 20 g portions were aseptically weighed into filter stomacher bags (SFB-0410; Spiral Biotech., Bethesda, MD) and inoculated with 1 ml of the appropriate dilution of *C. perfringens* cell suspension to obtain between 0 and 6 $\log_{10}$ CFU/g. Negative controls consisted of either meat samples inoculated with 1 ml of 0.1% (w/v) peptone-water with no bacterial cells or the enterotoxin-negative strains ATCC 3624 or FD-1. Each sample was diluted with 20 ml of filter-sterilized phosphate buffered saline (PBS, pH 7.4) containing 0.1% Tween 80 (Sigma Chemical Co., St. Louis, MO). The beef samples were homogenized for 1 min in a Stomacher Lab-Blender 400 (Tekmar Company, Cincinnati, OH). Meat homogenates (7–10 ml) were transferred to sterile 15-ml conical screw cap tubes (Sarstedt Inc., Princeton, NJ). One ml of the meat homogenate was transferred to 9 ml of CMM and incubated at 37°C. One ml aliquots were collected after 2 and 4 h of incubation in CMM for extraction of total chromosomal DNA before the PCR amplification step using a commercial G-NOME™ DNA isolation kit (Bio 101 Inc., La Jolla, CA). The precipitated DNA was resuspended in 100 μl of sterile distilled water and stored at −20°C until further use.

2.3. Bacterial enumeration

The cell numbers for the enterotoxin positive and enterotoxin negative strains were determined by plating serial dilutions of the corresponding meat supernatant fluid onto TSC agar. As described above, the diluted samples were plated in duplicate on TSC agar using a spiral plater (Spiral Systems, Model D, Cincinnati, OH), and also by inoculating 0.1 ml of the meat supernatant fluid onto TSC agar. The plates were overlaid with an additional 10 ml of TSC agar and incubated overnight at 37°C in anaerobic jars.

Elmer Cetus), 0.5 μM of each primer (INTPS/INTNS), and 500–850 ng template genomic DNA (NCTC 8239 strain) purified by the method of Saito et al. (1992). Thirty cycles of PCR amplification were run using the temperature cycling conditions described above. The amplified products were detected by electrophoresing 10–15 μl of the reaction mixture through a 2% agarose gel in 40 mM Tris-acetate, 1 mM EDTA (TAE: pH 8.0) followed by ethidium bromide staining (Sambrook et al., 1982). The concentration of the amplified products was also measured by absorbance at 260 nm (A_{260}).

2.7. Southern hybridization and detection of PCR products

The electrophoresed PCR products were transferred onto Nylon 66 Plus membranes (Hoefer Scientific Instruments, San Francisco, CA), by the method of Southern (1975), and baked at 80°C for 2 h in a conventional oven to fix the DNA. The membranes were then transferred to 50-ml conical centrifuge tubes (Sarsted Inc., Princeton, NJ) and hybridized to an internal 150 bp DIG-labeled probe in a volume $\geq$20 ml per 100 cm^2, at a final concentration of 20 ng/ml. The hybridization reaction was incubated overnight at 65°C. Next, the membranes were transferred to 15 × 100 mm petri plates (Fisher Scientific Co., Malvern, PA) for colorimetric development of the hybridization reaction using a DIG-DNA detection kit according to the manufacturer's instructions (Genius™ System User's Guide for Membrane Hybridization, Boehringer Mannheim, Indianapolis, IN). The substrate solution was pre-incubated at 37°C before development of the color reaction. The damp membranes were immersed in the substrate solution and incubated at 37°C in the dark with no agitation. Color development was monitored for 15–30 min. The reaction was stopped by washing the membranes with distilled water followed by a Tris-EDTA solution (10 mM Tris–HCl, pH 8.0, 1 mM EDTA). The stained membranes were stored in the dark.

2.8. Solution hybridization ELISA

Analysis of the amplified PCR products was assessed by two chemiluminescent assays using the 150 bp digoxigenin-labeled probe previously described or an alkaline phosphatase labeled oligonucleotide probe (AP). The latter consisted of a 15-mer oligonucleotide (Table 1) conjugated to an alkaline phosphatase enzyme at the 5′ terminal (GENOSYS Biotechnologies Inc.).

Capture of the PCR products hybridized to the digoxigenin or alkaline phosphatase probe was accomplished by the non-covalent interaction of the 5′ biotin group of the PCR product to streptavidin. Microlite™ 2 removawell® strips (Dynatech Laboratories Inc., Chantilly, VA) were coated with 5 μg of streptavidin (Boehringer Mannheim) in a volume of 50 μl/well using a carbonate coating buffer (0.6 M $NaHCO_3$, 0.6 M Na_2CO_3, pH 9.6). Plates were sealed and incubated overnight at 4°C. Before use, the plates were blocked for 1 h with 200 μl of buffer A (100 mM Tris–HCl, 150 mM NaCl; (pH 7.5), 2% (w/v) blocking reagent (Boehringer Mannheim).

For hybridization to the 150 bp digoxigenin-labeled probe, an aliquot of 10 μl (20%) of the PCR product was added to 40 μl of solution I (5 × SSC, 1% blocking reagent, 0.1% *N*-laurylsarcosine, 0.02% sodium dodecyl sulfate (SDS), 100 ng/ml herring sperm DNA (AMRESCO, Solon, OH), containing the 150 bp probe at a concentration of 1.27 ng/μl. The mixture was heated at 100°C for 5 min, allowed to cool to room temperature for 5 min, and then transferred to the streptavidin coated well. After addition of the samples, the plate was sealed and incubated at 37°C for 30 min. After incubation, the wells were washed 6 times with 200 μl of buffer B (0.05 M Tris–HCl, 0.001 M EDTA, 0.150 M NaCl (pH 7.5), 0.05% Tween 20) with no incubation. After washing, 50 μl of a 1:1000 dilution in buffer A, of an anti-digoxigenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim) were added, the plates were sealed, and incubated at 37°C for another 30 min. After incubation the plates were washed 6 times with buffer B and 200 μl of Lumiphos 530™ pre-warmed to room temperature was added. The chemiluminescent hybridization signals were immediately recorded using a Dynatech ML3000 luminometer (Dynatech Laboratories, Chantilly, VA).

For hybridization to the alkaline phosphatase-labeled probe, an aliquot of 10 μl (20%) of the PCR product was added to 30 μl of solution I. The mixture was heated to 100°C for 5 min and then transferred to ice. A volume of 10 μl solution I containing the AP-probe at a concentration of 2.65 ng/μl was added and the samples were transferred to the streptavidin coated wells. After addition of the samples, the plate was sealed and incubated at 37°C for 30 min. After incubation the wells were washed 6 times with 200 μl of buffer B with no incubation, and 200 μl of Lumiphos 530™ pre-warmed to room temperature was added. The chemiluminescent hybridization signals were immediately recorded using a Dynatech ML3000 luminometer. The luminometer readout parameters were set for a cycle mode with 20 plate reading cycles at 50 readings per well and a 30 s pause after each plate reading. The temperature of the plate carrier was set for 37°C with no mixing.

3. Results

A 750 bp sequence was selected as the target for PCR detection. Previous work with partial sequences within the enterotoxin A gene had shown its specificity for *C. perfringens* within a heterogeneous bacterial background flora (Baez and Juneja, 1995a,b). The temperature cycling conditions allowed for ~2 h amplification time due to short denaturation and annealing times. Amplification by PCR was performed on beef homogenates spiked with 10^{-1} through 10^{-7} dilutions of the overnight cultures which corresponded to 6, 5, 4, 3, 2, 1 and 0 $\log_{10}$ CFU/g. The homogenates were also used for anaerobic plate count on TSC to enumerate *C. perfringens* in the spiked beef samples.

A commercial chromosomal DNA extraction kit was used to increase the sensitivity of the detection assay within the shortest enrichment time possible (2–4 h). Efficient amplification was achieved when the total DNA present in the samples

was extracted and concentrated 10-fold relative to the original 1 ml sample (100 μl final volume). Fig. 1A and B show the representative amplification products from raw beef samples spiked with *C. perfringens*, resolved by ethidium bromide-stained agarose gel electrophoresis. Enrichment incubation for 2 h allowed detection of $\sim 3-4$ $\log_{10}$ CFU/g (Fig. 1A) whereas the 4 h incubation (Fig. 1B) provided detectable levels at or below 1 $\log_{10}$ CFU/g of freshly grown *C. perfringens* cells, within a heterogeneous bacterial background of $\sim 10^6$ CFU/g present in the raw ground beef. Similar performance of the PCR method was obtained when cooked beef samples (autoclaved 121°C/15 min) were spiked with enterotoxigenic *C. perfringens* strains (data not shown).

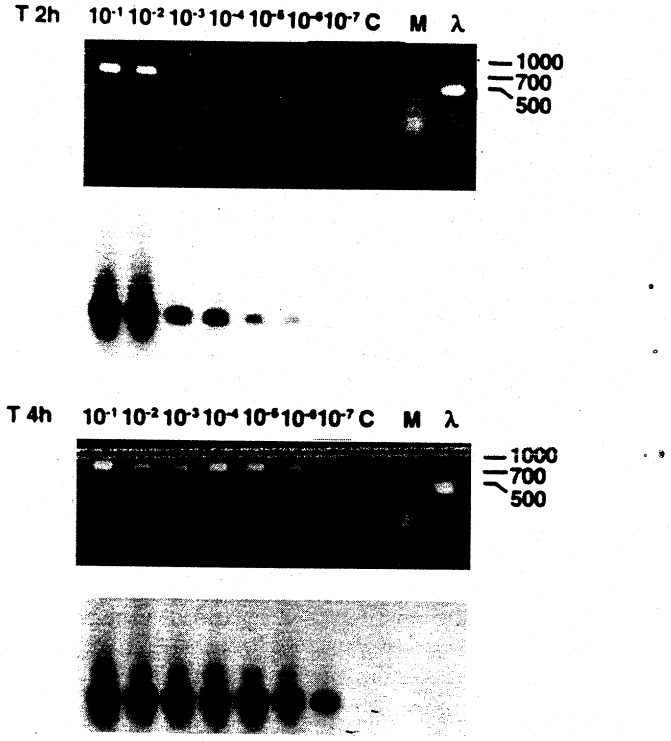

Fig. 1. Agarose gel electrophoresis (upper panel) and Southern blot analysis (lower panel) of PCR amplified products after 2 h, and 4 h enrichment in cooked meat medium (CMM). Primer set CPEPS/CPENS was used for amplification of a 750 bp sequence of the enterotoxin gene. The panel shown is representative of the amplification results after meat samples were spiked with $10^{-1}-10^{-7}$ dilutions of overnight cultures of enterotoxigenic *C. perfringens*. The meat supernatants analyzed averaged between 2.15×10^6 to 8.00×10^6 CFU/g of *C. perfringens* as determined by anaerobic plate counts. C, non-spiked beef control; M, BioMarker Low™ molecular weight marker; λ, Bacteriophage Lambda DNA amplification control.

Table 1
Synthetic oligonucleotide sequences

Oligonucleotide	Sequence[a] (5′ to 3′)	Location on the CPE gene
ENTPS	TGTAGAATATGGATTTGGAAT	426–446
ENTNS	AGCTGGGTTTGAGTTTAATGC	789–769
INTPS	CAAATGAATATGTATATTATAA	500–522
INTNS	ATATTTCCTAAGCTATCTGCAG	650–629
CPEPS	AGGAGATGGTTGGATATTAGG	267–287
CPENS	ATATTGTCCGGCATCTAAGCT	1017–997
AP probe	AP-GGAATATGGCTTAGT	607–621

[a]References: Saito et al. (1992); Van Damme-Jongsten et al. (1989).

Confirmation of the amplified PCR products was performed by hybridization to an internal DNA probe followed by signal development using an ELISA-based detection procedure. Biotinylation of the primer pair CPEPS/NS provided the capture of the hybridized PCR product onto the streptavidin-coated solid support. The oligonucleotide primer sequences (INTPS/INTNS) shown in Table 1 were used to generate a 150 bp DIG-labeled probe internal to the *C. perfringens* 750 bp PCR product. The sensitivity of the hybridization technique allowed for detection and confirmation of the amplification products with as little as 2 h of enrichment (Fig. 1). The DNA hybridization consistently provided a 10–100-fold higher level of sensitivity over ethidium bromide-agarose gel electrophoresis (Fig. 1). Cell numbers between 0 and 1 $\log_{10}$ CFU/g were consistently detected by anaerobic plate count within 2 h, corresponding to the highest level of detectable cells by Southern hybridization and DIG-ELISA. This level of sensitivity at 2 h was not obtained by agarose gel electrophoresis or the AP-ELISA. After 4 h incubation the anaerobic plate count was > 2 $\log_{10}$ CFU/g. Inconsistent amplification signals were detected when non-enriched, undiluted meat supernatants were assayed. The negative controls, which included uninoculated beef samples containing a bacterial background flora of ~ 6 $\log_{10}$ CFU/g (Fig. 1; control lane), and beef samples inoculated with 0–6 $\log_{10}$ CFU/g of enterotoxin negative strains (data not shown), showed no reaction as confirmed by the absence of a hybridization signal.

Detection of the amplified products by the ELISA hybridization procedures provided a faster confirmation of the PCR products, that ranged from 2 h for the alkaline phosphatase probe hybridization to 3 h for the digoxigenin probe. Development of the hybridization signal by a chemiluminescent reaction allowed for detection within 10 min (per 96-well plate). Either method constituted a significant improvement in response time over the Southern hybridization, which required ~ 24 h for completion. Either probe (DIG or AP-conjugated) was able to provide sensitive results as compared with the Southern hybridization procedure (Figs. 2 and 3). For the alkaline phosphatase conjugated probe (AP-ELISA), the maximum temperature tolerated by the probe was 60°C. This limitation precluded the denaturation of the PCR products in the presence of the probe, and therefore required the addition of the probe before transfer to the streptavidin coated well.

The DIG-ELISA did not have this limitation, since the digoxigenin-labeled probe withstood boiling without loss of activity. The only limitation was the additional anti-digoxigenin antibody step required to bring the alkaline phosphatase enzyme in contact with the hybridized probe-PCR product. This resulted in an additional 30 min incubation, plus an additional washing step before addition of the substrate. Besides the minor limitations mentioned above, both methods have the potential for automation in a PCR-ELISA detection assay.

The choice of substrates for development of the hybridization reaction contributed significantly to the overall rapidity of the assay. Use of colorimetric substrates such as *p*-nitrophenyl phosphate proved much slower for hybridization signals where the amount of PCR product was near the low sensitivity range shown

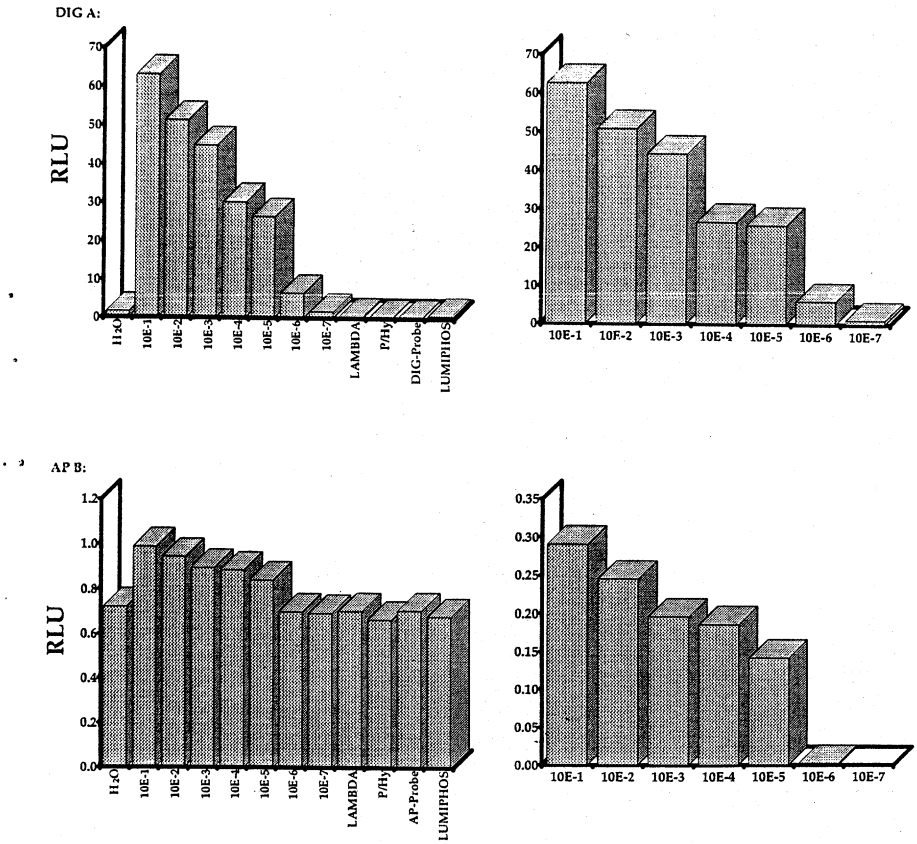

Fig. 2. Comparison of chemiluminescent detection of PCR products of *C. perfringens* after 2 h enrichment in CMM. Representative profiles for chemiluminescent detection using digoxigenin labeled probe (A) and the alkaline phosphatase conjugated probe (B) are shown for *C. perfringens* ATCC 10288. Graphs on the left include results for the controls included. Right side panels indicate background-subtracted results. RLU: relative light units.

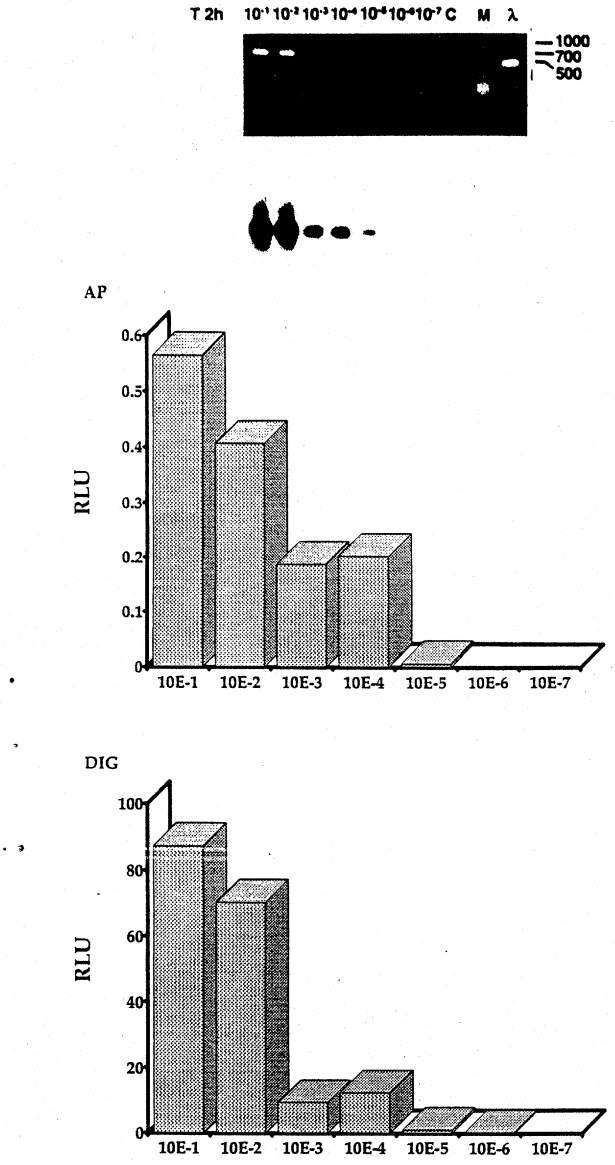

Fig. 3. Comparative analysis of PCR amplified products after 2 h enrichment in cooked meat medium (CMM). Representative results are shown for *C. perfringens* NCTC 8239. Agarose gel electrophoresis, Southern hybridization, and chemiluminescent ELISA (AP/DIG) are shown. C, non-spiked beef control; M, BioMarker Low™ molecular weight marker; λ, Bacteriophage Lambda DNA amplification control. RLU: relative light units.

by the Southern hybridization assay. Development of these solution hybridization reactions required times of ~ 1 h with a high incidence of negative results when background absorbance was subtracted (data not shown).

The nature of the probes used for each assay also had an influence in the signal obtained. Fig. 2 shows a comparison of the representative results for duplicate PCR products analyzed by both methods. The digoxigenin probe showed a higher signal-to-noise ratio than the alkaline phosphatase conjugated probe. These observations were consistent for all PCR products analyzed and resulted in a 10-fold lower sensitivity for the AP-ELISA.

A comparison of the three methods; ethidium bromide-stained agarose gel, Southern hybridization, and AP/DIG chemiluminescent ELISA is shown in Fig. 3. At the shorter enrichment incubation (2 h), the DIG-ELISA results were as sensitive as the hybridization results obtained by Southern hybridization, whereas the AP-ELISA showed a 10-fold lower sensitivity. Both ELISA detection methods provided a significant reduction in the response time when compared to Southern hybridization, while providing superior sensitivity as compared to agarose gel analysis. For the DIG-labeled probe generated by PCR amplification, the level of sensitivity is estimated at around 200 fg when applied in Southern hybridization assays. Terminally labeled oligonucleotides (5′/3′) consistently show a 10–100-fold lower sensitivity when applied in hybridization assays. These observations were consistently observed when the two probes were applied in an ELISA hybridization format.

4. Discussion

The frequent association of *C. perfringens* with outbreaks of food poisoning involving meat and poultry products (Granum, 1990; McClane, 1992) warrants the development of detection methodologies for these types of food matrices, while providing rapid identification in a sensitive and specific fashion. In the present study, detection of enterotoxigenic *C. perfringens* in beef was achieved by nucleic acid amplification of a portion of the enterotoxin A gene. The target gene was selected because of its association with pathogenicity in food poisoning outbreaks (Duncan et al., 1972; Frieben and Duncan, 1973; McDonel, 1979; Stark and Duncan, 1971; Walker, 1975) and its specificity for enterotoxigenic strains of *C. perfringens* within a heterogeneous background (Baez and Juneja, 1995a,b; Granum, 1990). Detection of the enterotoxin gene was accomplished by a combination of a short enrichment incubation and a nucleic acid extraction procedure. The enrichment increased the number of cells, and therefore the number of target sequences, thus improving the final sensitivity of the assay, and also reduced the inhibitors of the PCR assay that may be present in the undiluted meat homogenate (Rossen et al., 1992).

Confirmation of the PCR amplified products by Southern hybridization provided a higher level of sensitivity for the detection assay (estimated around 0.2 pg) while providing confirmation for the amplified DNA. Confirmation of the PCR products

by solution hybridization ELISA was demonstrated to be as reliable as the Southern hybridization assay in sensitivity and specificity. The ELISA protocol has the added advantage of providing confirmation of the results within 2–3 h after the PCR amplification procedure. When compared to a 96-well dot blot format for Southern hybridization, the solution hybridization ELISA also has the capability for handling a large number of samples. The substrate for development of the hybridization reaction contributes significantly to the overall rapidity of the assay. Development of colorimetric hybridization reactions required incubation times ranging from 15 min to ~ 1 h with a high incidence of negative results. Chemiluminescent substrates provided faster development of results. Signal intensity was superior for the digoxigenin labeled probe than for the alkaline phosphatase conjugated probe. The 10-fold higher sensitivity observed for the DIG-ELISA resided in the signal amplification brought in by the multiple incorporation of the digoxigenin molecule linked to the dUTP in the double stranded 150 bp probe. Addition of the anti-digoxigenin alkaline phosphatase conjugated antibody resulted in an amplified enzyme-substrate reaction hybridized to each strand of the PCR product. The AP-ELISA relied on a single alkaline phosphatase group per oligonucleotide probe, specific for only one of the DNA strands of the PCR product.

In conclusion, the PCR amplification assay developed in this study combined a short enrichment incubation with a chromosomal DNA extraction step preceding amplification. The added nucleic acid extraction step provided a purified target DNA within a heterogeneous DNA pool present in the extracted aliquot. Upon dilution, this template DNA constituted an ideal sample for PCR amplification as reflected by the superior amplification observed by agarose gel electrophoresis. The confirmation of the amplified PCR products was accelerated by the use of solution hybridization ELISA in a chemiluminescent detection system. Detection of *C. perfringens* in spiked beef samples demonstrated a higher level of sensitivity over conventional cultivation methods, while providing direct evidence for the presence of *C. perfringens* strains harboring the enterotoxin gene. The assay detected the clostridial enterotoxin A gene at levels below 1 $\log_{10}$ CFU/g of meat in the presence of the background flora ($\sim 10^6$ CFU/g) present in raw beef. It is also important to recognize that the cells used in this study were healthy, i.e. were grown under optimal growth conditions. The total time for enrichment incubations and the overall assay time may be affected by the physiological state of the cells if conditions of stress or cell injury prevail.

Overall, the assay required 2–4 h of enrichment incubation, followed by a 2 h sample extraction step, 2 h of PCR amplification, and finally 2–3 h for chemiluminescent ELISA confirmation. The entire sample analysis required ~ 12 h to complete when sample preparation and the final detection step were taken into consideration. The technique holds promise due to its capability of handling a large number of samples, its specificity and its sensitivity.

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